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(71) Applicant *(for all designated States except US)*: ZENECA LIMITED [GB/GB]; 15 Stanhope Gate, London W1Y 6LN (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: DUCHARS, Matthew, Guy [GB/GB]; 15 Victoria Embankment, Darlington, Co Durham DL1 5JR (GB). TOPPING, Andrew, William [GB/GB]; 18 Silver Meadows, Barton, Richmond, North Yorkshire DL10 6SL (GB).

(74) Agents: LOCKE, Timothy, John; Zeneca LifeScience Molecules, Intellectual Property Group, Belasis Avenue, P.O. Box 2, Billingham, Cleveland TS23 1YN (GB) et al.

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(57) Abstract

Plasmids which comprise: (1) an origin of replication; (2) an additional sequence required for plasmid replication or preferably a gene giving a selective advantage; and (3) two expression cassettes each of which are located between (1) and (2) but are separated by (1) and (2) from each other; and which are free from inverted repeat sequences (other than in (3)) are highly persistent though successive generations of microbes containing them.

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PRODUCTION OF PROTEINS, PLASMIDS CODING THEREFOR AND ORGANISMS CONTAINING SUCH PLASMIDS

THIS INVENTION relates to the production of proteins, plasmids coding therefor and organisms containing such plasmids.

It is known to modify microbes to produce desired proteins, for example enzymes, by incorporating plasmids coding for a desired protein into a microbe which would not otherwise produce it or which would not produce it in sufficient quantity. There is however a tendency for the plasmids to be lost on prolonged cultivation of the organism. It is believed that this is at least partly due to the production, on each division of the microbes, of a small number of daughter cells which contain none of the said plasmids and which are in consequence at a selective advantage over those which contain the desired plasmids. In the course of time, cells which contain none of the desired plasmids increase as a proportion of the total cells present.

In order to overcome this effect it is known to incorporate in the plasmid one or more genes which give a selective advantage to the microbe, for example genes giving resistance to an antibiotic are suitable if the microbes are cultivated in the presence of the antibiotic, or genes making good a deficiency in the host organism may be incorporated.

There is also a tendency for mutated variants of the plasmid which do not produce the desired protein to be produced for example by a mutation leading to the introduction of a stop codon in the gene or partial or complete deletion of the protein coding sequence.

Microbes containing such mutated plasmids will tend to have a selective advantage compared with those having the original plasmid and the desired protein producing capability of the microbe may be reduced or lost on prolonged cultivation.

In DDR patent 233,851 A1 there are disclosed vector plasmids in which a sequence is present twice in opposite senses (inverted repeat sequences) into each of which sequences duplicate genes are cloned or recloned. This is said to cause increased synthesis of the gene product in the microorganism due to the gene dosage effect. The inverted repeat sequences must each have at least one homologous cleavage site into which the duplicate genes may be inserted. Stable plasmids are disclosed as producible.

Surprisingly we have now found that in certain cases stable plasmids can be produced from vectors which do not have inverted repeat sequences. This has important advantages.

Firstly, it reduces or eliminates the number of homologous cleavage sites and therefore the tendency to cut the plasmid into ineffective fragments in the process of cutting it to insert the desired gene. It is clear that each inverted sequence of DDR 233851 A1 must have at least one cleavage site and that these will be homologous. Normally the vector plasmids will be cleaved at both such sites. This increases the difficulty of constructing a plasmid with the desired gene correctly inserted into both of the inverted sequences. For example the free ends may link with a single added gene leaving plasmids with only one such gene. In addition

non-functional nucleic acid fragments will be formed. The greater the number of cleavage sites in the inverted sequences the greater this problem becomes.

Secondly, it reduces the dangers of inter- and intra-plasmid recombination which can lead to scission of the plasmid. The presence of unnecessary DNA adjacent to the desired genes increases the likelihood of recombination leading to the loss of part or all of that gene. In addition, recombination between the inverted repeat sequences in different plasmid molecules (copies) may lead to formation of unstable multimeric plasmids.

By "Expression cassette" is meant a DNA sequence effective in production of a protein which comprises a promoter sequence and ribosomal binding site, a gene coding for a protein and normally a termination sequence. The gene may code for a fusion protein and may have a signal sequence.

Thirdly, it enables the production of smaller plasmids. We have found that according to our invention plasmids (excluding the desired expression cassette) of at most 10kB, for example at most 6kB and even for example at most 5kB may be used. Each such plasmid places a smaller burden on the host organism than a larger plasmid. They normally also have higher copy numbers than larger plasmids for example 50-300 or even more than 300 plasmids per host cell, with, in general, an improvement in output of the desired protein.

Fourthly, the inverted repeat sequences may themselves code for proteins thus placing an additional burden on the host and complicating separation of the desired product.

The invention comprises a continuous process for the production of organisms containing plasmids or polypeptides expressed by genes of such plasmids in which a plasmid comprises an origin of replication and an additional sequence required for plasmid replication and/or preferably a gene giving a microbe a selective advantage, and two expression cassettes each being in a DNA sequence located between the originating sequence and the gene giving the selective advantage or an additional sequence required for plasmid replication but being separated from one another on one side by DNA comprising the origin of replication and on the other side by DNA comprising the gene giving the selective advantage or the sequence required for plasmid replication, the plasmid being substantially free from inverted repeat sequences other than sequences represented by the expression cassettes.

If desired, additional expression cassettes may be included. The expression cassettes may be different but preferably are the same.

Preferably at most one antibiotic resistance gene is present.

Preferably the expression cassettes code for the same protein or for proteins which are enzymes used together for catalytic purposes whereby the organism or material derived therefrom is useful as a catalyst or a component of a catalyst comprising both such enzymes.

By "continuous process" is meant a process in which fresh nutrients are added and product removed continuously or intermittently without discontinuing the fermentation. Before operating a continuous process it

is necessary to reach a suitable concentration of the organisms per unit volume of culture medium. It is normal to reach that condition by inoculation of a fermenter with the desired organism and permitting it to grow until the desired concentration is reached, and no product would normally be removed until then other than for sampling. It is preferred that at least 5, preferably at least 10 and more preferably at least 50 generations of organisms be produced after this has been achieved. Suitably a production of 5%, preferably 10% and more preferably at least 15% by weight of the desired protein is produced based on the total protein content of the organism.

The invention also comprises a plasmid which comprises an origin of replication and an additional sequence required for plasmid replication and/or preferably a gene giving a microbe a selective advantage, and two expression cassettes which express the same polypeptide or different polypeptides which are enzymes used for catalytic purposes and which are preferably the same, each being in a DNA sequence located between the origin of replication and the gene giving the selective advantage or the additional sequence required for plasmid replication but being separated from one another on one side by DNA comprising the origin of replication and on the other side by DNA comprising the gene giving the selective advantage or the additional sequence required for plasmid replication, the plasmid being substantially free from inverted repeat sequences other than sequences represented by the expression cassettes. A second gene giving a selective advantage may be present if desired as

also may additional expression cassette(s).

It will be apparent that if a recombination occurs between homologous sequences of DNA from the two expression cassettes leading to deletion of the intervening sequence that sequence must comprise either the origin of replication or the sequence required for plasmid replication (in which case the plasmid will not replicate further) or the gene giving the selective advantage in which case the plasmid will tend to be lost by selection. Such losses however will be less than those occurring in plasmids with inverted repeat sequences in addition to the genes.

Surprisingly we have found that in the case of plasmids in which the expression cassettes are close together, for example separated by at most 5kB and preferably at most 2kB (as judged by the separation of their closest points) if one of the cassettes is deleted the resulting plasmid is at a selective disadvantage and therefore the microbes containing plasmids with both active cassettes continue to predominate until simultaneous inactivation of both cassettes occurs.

In a further form of the invention the genes for producing the desired protein are arranged to read in the opposite sense in the plasmid, i.e. if one is regarded as clockwise the other is anticlockwise. This makes it difficult for homologous sequences to come into contact, especially if the genes are close together, in a "head-to-head" or "tail-to-tail" relationship with as little DNA between them as possible.

The invention also comprises microbes containing plasmids according to the invention and also processes in

which a desired protein is produced by means of such plasmids and/or microbes. The protein may be separated therefrom for example in a purified form if desired.

The invention also comprises a process in which a plasmid is cut at a first restriction site, a desired expression cassette is inserted at the first restriction site to form a modified plasmid, the modified plasmid is cut at a second restriction site which is not homologous with the first restriction site and a desired expression cassette is inserted at the second restriction site, the first and second restriction sites being separated on one side by a sequence which comprises an origin of replication and if they are not separated on the other side by a sequence which includes a gene giving a selective advantage to the host organism or a sequence required for plasmid replication, such a gene is inserted on that side, the plasmid being substantially free from inverted repeat sequences other than sequences represented by the expression cassette.

Preferably the host has a recombinational deficiency. The deficiency may be in consequence of the non-functionality or deletion of a recombination gene, for example rec A or preferably rec J gene.

Increased Stability of Xylanase Expressing Plasmid In Continuous Culture

Plasmid pSPR6 (see Figure 1), (in Escherichia coli NM554 host NCIMB 40786 deposited with The National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB2 1RY Scotland UK on 8 February 1996 under the Budapest Treaty) contains unique NotI and SpeI restriction sites which are positioned on

either side of the plasmid origin of replication (ori) and on either side of a tetracycline resistance marker tetA/R. This plasmid allows the insertion of two independent copies of an expression cassette to improve the genetic stability of the plasmid during fermentations.

In this example the expression cassette was DNA encoding the expression of a fungal xylanase comprising of a constitutive promoter (gene A3 promoter from bacteriophage T7), a ribosome binding site (from lac z of E.coli), the coding sequence for the enzyme (truncated xylanase gene from plasmid pNX10 described in WO 93/25693) and a transcriptional terminator from bacteriophage T4. The expression cassette was flanked by either NotI or SpeI restriction sites. The expression cassette can be obtained by complete digestion of the plasmid pSPR8 in E. coli NM554, (NCIMB 40787 deposited with The National Collections of Industrial and Marine Bacteria Limited, 23 St Machar Drive, Aberdeen AB2 1RY Scotland UK on 8 February 1996 under the Budapest Treaty) using restriction endonucleases NotI or SpeI in a high salt restriction buffer.

Strain E.coli NM554 (pSPR8) was constructed as follows: Plasmid DNA was prepared from E. coli strain NM554 (pSPR6) grown overnight at 37°C in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) using "Rapid Pure Miniprep" (RPM) (Stratech Scientific Ltd, Luton, UK) following the manufacturers protocol. Plasmid DNA may also be isolated using standard methods such as described by Sambrook et al[1]. 50µl of plasmid pSPR6 DNA was digested with restriction endonuclease NotI (Boehringer

Mannheim, Lewes, UK) with the addition of 6µl of the manufacturers H buffer (high salt restriction buffer) and 20 units of restriction enzyme. Digestion was carried out at 37°C for 16h.

The xylanase expression cassette was digested with NotI and ligated to plasmid pSPR6 similarly digested with NotI, as follows. The two species of DNA were mixed and the restriction enzyme and other contaminants were removed using an RPM miniprep (following the manufacturer's protocol but with the following modifications: DNA mixture used in place of cleared lysate and DNA eluted into 40µl). 0.5µl of 100mM adenosine triphosphate (ATP) was added with 4µl of M buffer (medium salt restriction buffer). 1 unit of T4 DNA ligase (Boehringer Mannheim) was added and the reaction incubated at 18°C for 16h.

5µl of the ligation reaction was mixed with 100µl of E.coli strain JM109 (ATCC 53323) competent cell suspension, produced by calcium chloride treatment essentially as described by Hanahan[2], and incubated on ice for 45m. Cells were then heat shocked at 42°C for 90s and returned to ice for 2m. 1ml of L broth was added and cells incubated at 37°C, with shaking, for 1h before plating out dilutions onto L agar plates (L broth + 1% bacteriological agar) containing 10µg/ml tetracycline and 1% remazol brilliant blue - xylan (RBB-Xylan, Sigma, Poole, UK). Plates were incubated for 24h at 37°C. One colony which gave a zone of clearing and contained the expected plasmid when miniprep DNA was digested with NotI was designated JM109 (pSPR7).

Plasmid DNA from pSPR7 which is shown

diagramatically in Figure 2 was prepared as above from an overnight culture of JM109 (pSPR7) in L broth supplemented with 10µg/ml tetracycline. 50µl of plasmid DNA was linearised by digestion with restriction endonuclease SpeI (Boehringer Mannheim) by adding 5µl of manufacturers H buffer and 20 units of SpeI enzyme. Reaction was incubated for 16h at 37°C. The position of the NotI flanked xylanase expression cassette (XYL) relative to the plasmid origin of replication (ori) and the selectable marker (tetA/R) is shown.

A second xylanase expression cassette, identical to the first except flanked by SpeI restriction sites rather than NotI, was digested with SpeI as described above. This expression cassette may be obtained by the complete digestion of plasmid pSPR8 with the restriction enzyme SpeI. This DNA was ligated to SpeI digested plasmid pSPR7 exactly as described above but with selection of transformants on L agar containing 10µg/ml tetracycline.

Tetracycline resistant colonies were screened using a rapid lysis method (Twigg and Sherrett[3]) to estimate the size of the plasmid carried. 1 colony which contained a plasmid larger than plasmid pSPR7 and which subsequent digests of isolated plasmid DNA with NotI and SpeI restriction endonucleases showed to contain 2 copies of the xylanase expression cassette was designated JM109 (pSPR8), see Figure 3 which shows plasmid pSPR8 showing the positions of both inserted xylanase expression cassettes (XYL) relative to the plasmid origin of replication (ori) and selectable marker (tetA/R).

Plasmid DNA was prepared from JM109 (pSPR8) and JM109 (pSPR7) and 1µl used to transform E.coli strain

NM554 (Stratagene, Cambridge, UK). Preparation of DNA and competent cells and transformation of DNA were done as described above. Transformants were selected on L agar containing 10µg/ml tetracycline. Stocks of NM554 (pSPR7) and NM554 (pSPR8) were stored at -70°C in L broth containing 10µg/ml tetracycline and 25% (v/v) glycerol.

To prepare a fermentation inoculum, 50ml of L broth containing 10µg/ml tetracycline was inoculated with 500µl from freezer stock and grown for approximately 4h at 37°C with rapid aeration before transfer to the fermenter.

Fermentations were done using Braun ED/ER5 fermenters (B. Braun Biotech, Reading, UK). Vessels were in situ sterilised and bottom agitated using 2x70mm diameter Rushton impellers. The fermenter working volume was approximately 2L. The medium used throughout the experiments was JV1 (see appendix). Temperature was maintained at 37°C ± 0.2°C. The pH was measured using an Ingold pH probe and maintained at 6.7 ± 0.1 by the controlled addition of filter sterilised 10M NH_4OH and 2M H_3PO_4. An agitation speed of 600 rpm was used with air or 35% O_2 aeration to maintain a $\%pO_2$ (dissolved oxygen tension) between 20% and 80%, of saturation measured by an Ingold oxygen probe. Foaming was controlled by the addition of sterile Diamond Shamrock PPG Foamaster EEA 142 at a rate of 0.1ml/h.

50ml inoculum was transferred to the fermenter which contained 2L of JV1 medium plus 30g/l glycerol and 10ppm Fe^{2+} (as $FeSO_4.7H_2O$). Cultures were allowed to grow in batch to a point at which the CO_2 evolution rate was between 10 and 40mM/l/h (typically 20mM CO_2/l/h), when the fermenter was switched to continuous operation at a

dilution rate of $0.1h^{-1}$. JV1 medium was fed with separate feeds of sterile glycerol (feed rate 30g/l) and $FeSO_4.7H_2O$ (feed rate 10mg Fe^{2+}/l). 1ml samples were regularly withdrawn and stored at -20°C in 25% glycerol for later analysis of plasmid content. Enzyme activity and dry cell weight was also periodically measured in 10ml samples. Xylanase enzyme activity was determined by measuring the amount of reducing sugar released from soluble oat spelt xylan substrate (1%), essentially as described by Kellett et al[4]. The production of xylanase and molecular weight was confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on an 8-25% gradient gel with commasie blue protein staining using Phast electrophoresis system (Pharmacia Biotech, St Albans, UK). Dry cell weights were determined by pelleting the cells in the withdrawn sample by centrifugation at 5700 rpm in a Beckman TJ-6 centrifuge for 20m and resuspending cells in 2-3mls Tris buffer (100mM, pH7.2). Cells were then re-pelleted and dried in a pre-weighed tube in an oven at 105°C for 16h and mass of dried cells determined.

Changes to plasmid size during the fermentations were detected by plating of stored fermentation samples onto L agar containing 10µg/ml tetracycline and 1% RBB-Xylan. Colonies were then picked and lysed using the procedure described by Twigg and Sherrett[3]. Changes in plasmid size were seen as a shift in mobility of the plasmid band on a 1% agarose gel compared to the control plasmid (agarose gel electrophoresis was done as described by Sambrook et al[1]).

Figure 4 shows xylanase activities (KU/ml) for fermentations of E.coli strains NM554 (pSPR7) ◊, and

NM554 (pSPR8), ♦, growing in JV1 medium. Cultures switched from batch to continuous culture, with dilution rate of $0.1h^{-1}$, at approx. 10 hrs. These results show that strain NM554 (pSPR7), with a single copy of the xylanase expression cassette, very rapidly lost enzyme activity during continuous culture. SDS PAGE analysis of samples confirmed that the loss of enzyme activity corresponded to the loss of production of a heterologous protein band. Examination of plasmids using the rapid lysis technique showed no difference in plasmid size compared to pSPR7 control in samples taken up to 19 hrs, but showed several differently sized plasmids at the 50 hr and 72 hr sample points, indicating rearrangements and deletions had occurred in this plasmid causing the loss of enzyme activity.

Strain NM554 (pSPR8), containing 2 copies of the xylanase expression cassette, produced a high level of xylanase activity for 480 hrs in continuous culture. SDS PAGE confirmed production of heterologous protein over this time period. Analysis of plasmids from fermentation samples showed no detectable change in plasmid size during the course of this experiment, indicated that no rearrangements or deletions had occurred.

It was noted that the peak xylanase activity for NM554 (pSPR7) was higher than for NM554 (pSPR8) when the continuous fermentation was operated at a dilution rate of $0.1h^{-1}$. In order to investigate whether the observed increase in strain stability was due to this initial reduction in enzyme expression strain NM554 (pSPR7) was fermented as previously but with a dilution rate of $0.2h^{-1}$. This had the effect of reducing the peak xylanase

activity to a comparable level to that seen with strain NM554 (pSPR8) operated at a dilution rate of $0.1h^{-1}$. Results showed the same instability despite the reduced expression level. Analysis of plasmid content showed changes in plasmid size similar to those seen previously.

Increased Stability of Dehalogenase Expressing Plasmid in Continuous Culture

The invention was further exemplified through production of a second protein, a haloalkanoic acid dehalogenase. In this example the host strain, promoter, ribosomal binding site, structural gene and fermentation medium were all different from those used in the xylanase example indicating the wide applicability of the invention.

Plasmid pSPR6 was modified to remove the unique NotI restriction site and introduce a unique PstI restriction site at the same position. Plasmid pSPR6 DNA was isolated and digested with NotI restriction enzyme, as above. Approx 2µg of a synthetic oligonucleotide with the sequence GGCCCTGCAG was self annealed by heating to 94°C in high salt restriction buffer and cooling slowly to room temperature. The annealed oligonucleotide and digested pSPR6 DNA were mixed, ATP added to final concentration of 1mM and 1 unit of T4 DNA ligase added. Reaction was incubated at 18°C overnight. Ligation mix was transformed into JM109 competent cells as described above and plated onto L agar plates containing 10µg/ml tetracycline. Randomly picked colonies were screened by isolating plasmid DNA and digesting with Not I and PstI restriction enzymes in separate reactions. One clone which failed to digest with NotI but digested with PstI

was isolated and designated JM109 (pSPR6pst).

Plasmid pSPR6pst DNA was isolated and digested with PstI restriction enzyme in high salt restriction buffer. After 3h incubation at 37°C 1 unit of calf intestinal alkaline phosphatase (Boehringer Mannheim) was added to prevent relegation. The reaction was incubated at 37°C for a further 30 mins then stopped by adding EDTA to a final concentration of 5mM and heating the reaction to 75°C for 10 mins.

The dehalogenase expression cassette, comprising the E. coli trp promoter, a two cistron type ribosomal binding site (Gold and Stormo[5]), the hadD dehalogenase structural gene (Barth et al[6].,) and T4 phage transcriptional terminator, may be obtained by digesting the plasmid pSPR11.1 (deposited in E.coli host strain XL1 Blue MR at NCIMB as deposit no 40859 on 12 February 1997) with the restriction enzyme PstI as described above. Plasmid pSPR10, which contains a single copy of the dehalogenase expression cassette, may then be obtained by ligation of this DNA with the pSPR6pst DNA prepared as above, selecting for transformants on Lagar containing 10µg/ml of tetracycline.

Plasmid pSPR11.1 (available from NCIMB in host strain XL1 Blue MR, deposit no 40859), contains 2 copies of the same dehalogenase expression cassette. This plasmid was constructed by the addition of a second copy of the expression cassette into a unique SwaI restriction site on plasmid pSPR10. Plasmid pSPR10 DNA was isolated as previously and digested with restriction enzyme SwaI in high salt restriction buffer before treatment with calf intestinal alkaline phosphatase as described above.

The dehalogenase expression cassette may be obtained by digestion of pSPR11.1 with restriction enzyme PstI. To produce blunt ended DNA compatible with the SwaI digested plasmid pSPR10, 1 unit of T4 DNA polymerase (Boehringer Mannheim) and a final concentration of 200µM each deoxyadenosine 5' triphosphate, deoxy-cytidine 5' triphosphate deoxyguanosine 5' triphosphate and thymidine 5' triphosphate (all from Boehringer Mannheim) was added to the reaction after 3 h incubation and incubated a further 30 mins at 12°C. The reaction was stopped by heating to 75°C for 10mins. The blunt ended dehalogenase expression cassette and SwaI digested pSPR10 were ligated and transformed into XL1 Blue MR competent cells (Stratagene) as described above and plated onto L agar plates containing 10µg/ml tetracycline. Plasmid pSPR11.1 was identified by restriction digests of isolated plasmid DNA, as containing 2 copies of the expression cassette in opposite orientations to one another.

Fermentation experiments were conducted in a derivative of E.coli strain W3110 (ATCC 27325)(American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, USA) engineered to be recombinationally deficient due to a deletion of the recJ gene. The construction of this strain is given, but other recombinationally deficient hosts may also be used. 2 PCR products were produced of regions of the E.coli chromosome flanking the recJ gene, primers used were: CTGGATCCCGGCGTTTTCAGGCTTTGCTC with ACAGATCTTCACCGACCACAATAATCCGC to give product 1 and ACAGATCTTGACCCTGTGCGAGAAACTGG with TGGGATCCGCTCGGCGTTTACTTCTTCCA to give product 2. PCR

reactions were carried out using 35 cycles of denaturation at 94°C for 1 min; prime annealing at 60°C per 1 min and product extension at 72°C per 1 min. Reactions were performed in a volume of 100µl, containing 200µM of each nucleoside triphosphate. Taq DNA polymerase buffer (Promega, Southampton;) and 5 units of Taq DNA polymerase. E.coli W3110 cells were used as template DNA. The 2 PCR products produced were purified using an RPM column, as above, and cloned using the pMOS blue T vector kit (Amersham, Amersham, UK) following the manufacturers protocol.

Plasmid containing PCR product 1 was isolated and digested with restriction enzyme BglII (Boehringer Mannheim) in medium salt restriction buffer. A DNA fragment encoding streptomycin and spectinomycin resistance was produced by Bam HI restriction digestion of plasmid pUT::miniTn5Sm/Sp (De Lorenzo[7]) in medium salt restriction buffer. This fragment was ligated to the digested plasmid and transformed into XL1 Blue MR competent cells with selection for transformants on L agar containing 50µg/ml ampicillin and 25µg/ml streptomycin. The insert from this plasmid was released by restriction digestion with the enzyme BamHI in medium salt restriction buffer. This fragment was ligated to the plasmid containing PCR product 2 which was digested with the restriction enzyme BglII. Ligation and transformation was as above. A clone was identified by restriction digestion of isolated plasmid DNA in which the streptomycin/ spectinomycin resistance gene was flanked by DNA which normally flanks the recJ gene on the E.coli chromosome.

The deletion of the recJ gene on the E.coli chromosome was achieved by transformation of E.coli strain JC7623 (ATCC 47002). The above plasmid was digested using the restriction enzyme BamHI and the DNA concentrated by ethanol precipitation (Sambrook et al[1]) to give a final concentration approx 500ng/µl. Fresh electrocompetent JC7623 cells were produced (Sambrook et al[1]) and 2µl of digested plasmid DNA was transformed by electroporation in Gene Pulser Electroporation apparatus (Bio-Rad, Hemel Hempstead, UK) (15KV/cm, 25µF capacitance, 200Ω parallel resistance). After a 2hr recovery period shaking in L broth at 37°C, transformants were recovered on L agar containing 25µg/ml streptomycin and 25µg/ml spectinomycin. Transformants were screened for sensitivity to ampicillin by plating onto L agar containing 100µg/ml ampicillin. A transformant was identified as resistant to streptomycin and spectinomycin but sensitive to ampicillin and with an increased sensitivity to UV light compared to strain JC7623. This strain was designated JC7623 ΔrecJ.

The ΔrecJ mutation was introduced to strain W3110 by P1 phage transduction. A phage lysate was raised on JC7623 and used to infect W3110 using the method described by Miller[8]. Transductants were selected on L agar containing streptomycin and spectinomycin as above.

For fermentations the strain W3110 ΔrecJ was transformed with the plasmids pSPR10 and pSPR11.1 using electroporation as described above.

Fermentation inocula and fermentations of dehalogenase producing strains were carried out essentially as described for xylanase production except

that no yeast autolysate was present in the medium and glucose was used rather than glycerol.

Dehalogenase enzyme activity was measured as the rate of dechlorination of 2-chloropropionic acid, (Fluka Chemical, Gillingham, Dorset, UK) neutralised with NaOH.

Results of continuous fermentations of W3110 ΔrecJ (pSPR10) and W3110 ΔrecJ (pSPR11.1) are shown in figure 5. They show dehalogenase activities (Units/ml) for fermentations of E.coli strains W3110 ΔrecJ (pSPR10) ▲ and W3110 ΔrecJ (pSPR11.1) ● growing in JV1 medium, modified as described. The cultures were switched from batch to continuous operation, with dilution rate of $0.1h^{-1}$, at approx 40 hrs. It can clearly be seen that the strain with pSPR11.1, containing 2 copies of the dehalogenase expression cassette, has much greater stability than the strain with plasmid pSPR10, which has only a single copy of the expression cassette. It is clear that although the peak enzyme activity is reduced the overall productivity of the fermentation is greatly enhanced.

Appendix

Fermentation Medium (JV1)

K_2SO_4	2g/l
$MgSO_4.7H_2O$	1.5g/l
H_3PO_4 (85%)	0.14ml/l
$CaCl_2.2H_2O$	0.11g/l
Trace Elements Solution	1ml/l
Yeast autolysate (Biospringer, Low Salt, Grade D,)	20g/l
Thiamine HCl (Sigma Chemicals, UK 32g/l sterile stock)	0.5ml/l
Tetracycline hydrochloride (Sigma Chemicals, UK 67mg/ml sterile stock)	0.15ml/l

Trace Element Solution contained 0.2g/l $AlCl_3.6H_2O$, 0.08g/l $CoCl_2.6H_2O$, 0.02g/l $CuCl_2.2H_2O$, 0.01g/l H_3BO_4, 0.2g/l KI, 0.5g/l $MnSO_4.H_2O$, 0.01g/l $NiSO_4.6H_2O$, 0.5g/l $Na_2MO_4.2H_2O$, 0.5g/l $ZnSO_4.7H_4O$.

All chemicals were of "AR" grade and obtained from Fisons (Loughbrough, UK) unless otherwise stated. Sterilisation was carried out at 121°C for 30m.Thiamine, trace elements and tetracycline solutions were filter sterilised through a 0.2µm filter and added aseptically.

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CLAIMS

1 A continuous process for the production of organisms containing plasmids or polypeptides expressed by genes of such plasmids in which a plasmid comprises

(1) an origin of replication; and

(2) an additional sequence required for plasmid replication and/or preferably a gene giving a microbe a selective advantage; and

(3) two expression cassettes each being in a DNA sequence located between the originating sequence and the gene giving the selective advantage or the additional sequence required for plasmid replication; but being separated from one another on one side by DNA comprising the origin of replication and on the other side by DNA comprising the gene giving the selective advantage or the sequence required for plasmid replication;

the plasmid being substantially free from inverted repeat sequences other than sequences represented by the expression cassettes.

2 A process as claimed in Claim 1 in which the expression cassettes code for the same polypeptide and are preferably the same.

3 A process as claimed in Claim 2 in which the genes for producing the desired protein are arranged in the opposite sense in the plasmid.

4 A process as claimed in any preceding claim in which the host has a recombinational deficiency.

5 A process according to any preceding claim in which the plasmid is of at most 10kB (kilobase pairs).

6 A process according to any preceding claim in which the cassettes are separated by at most 5kB and preferably by at most 2kB as judged by the separation of their closest ends.

7 A process according to any preceding claim in which the polypeptide is a xylanase or dehalogenase.

8 A plasmid which comprises

(1) an origin of replication; and

(2) an additional sequence required for plasmid replication and/or preferably a gene giving a microbe a selective advantage; and

(3) two expression cassettes expressing the same polypeptide or different polypeptides which are enzymes used together for catalytic purposes which are preferably the same each being in a DNA sequence located between the originating sequence and the gene giving the selective advantage or an additional sequence required for plasmid replication but being separated from one another on one side by DNA comprising the origin of replication and on the other side by DNA comprising the gene giving the selective advantage or the sequence required for plasmid replication;

the plasmid being substantially free from inverted repeat sequences other than sequences represented by the expression cassettes.

9 A plasmid as claimed in Claim 8 in which the genes for producing the desired protein read in the opposite sense and have little DNA between their closest ends.

10 Plasmids adapted for use according to any of Claims 1 to 7.

11 Plasmids pSPR6 and pSPR8 as deposited in E.coli under numbers NCIMB 40786 and NCIMB 40787 and plasmid pSPR11.1 as deposited in E.coli under number NCIMB 40859 with The National Collections of Industrial and Marine Bacteria Limited on 8 February 1996 for NCIMB 40876 and NCIMB 40878 and on 12 February 1997 for NCIMB 40859 under the Budapest Treaty.

12 A process in which a plasmid is cut at a first restriction site, a desired expression cassette is inserted at the first restriction site to form a modified plasmid, the modified plasmid is cut at a second restriction site which is not homologous with the first restriction site and a desired expression cassette is inserted at the second restriction site, the first and second restriction sites being separated on one side by a sequence which comprises an origin of replication and if they are not separated on the other side by a sequence which includes a gene giving a selective advantage to the host organism or a sequence required for plasmid replication, such a gene is inserted on that side, the plasmid being substantially free from inverted repeat sequences other than sequences represented by the expression cassettes.

13 An organism which comprises a plasmid as claimed in any of Claims 8 to 11.

14 A plasmid as claimed in any of Claims 8 to 11 or made by a process as claimed in Claim 12 which has

at most one antibiotic resistance gene.

15 A process as claimed in any of Claims 1 to 7 in which the plasmid has at most one antibiotic resistance gene.

FIGURE 1 of 5

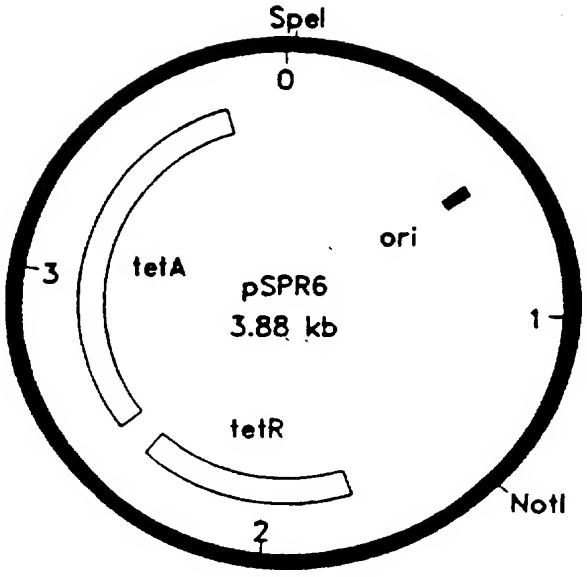


FIGURE 2 of 5

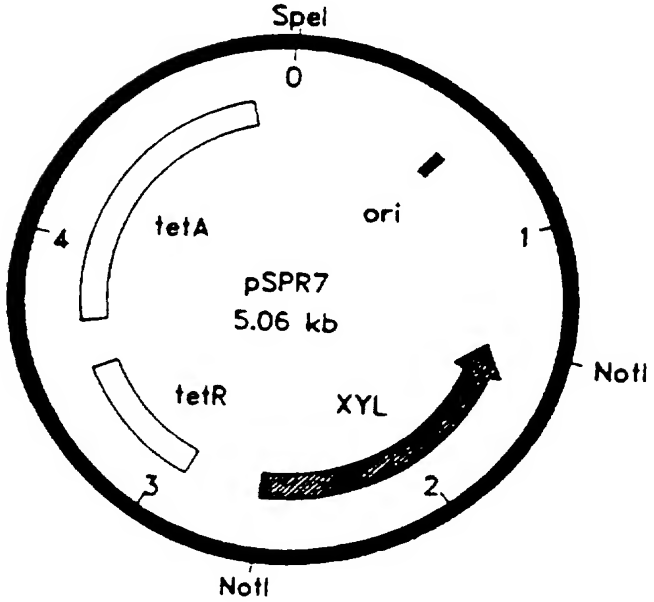


FIGURE 3 of 5

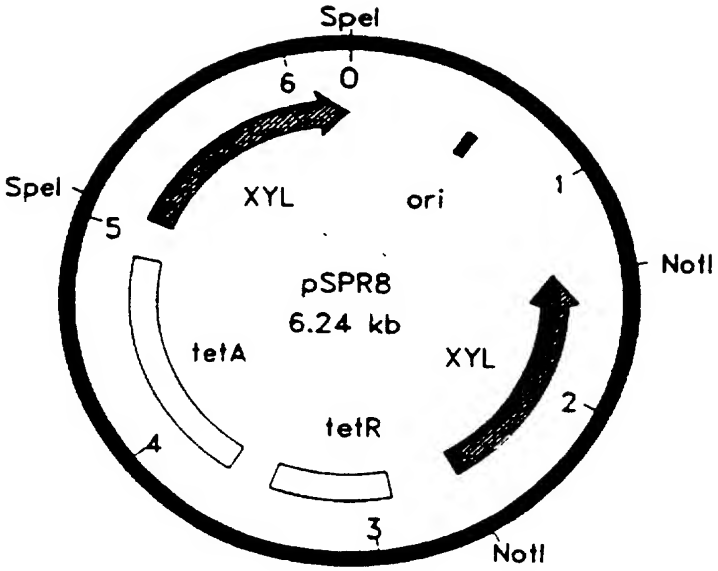
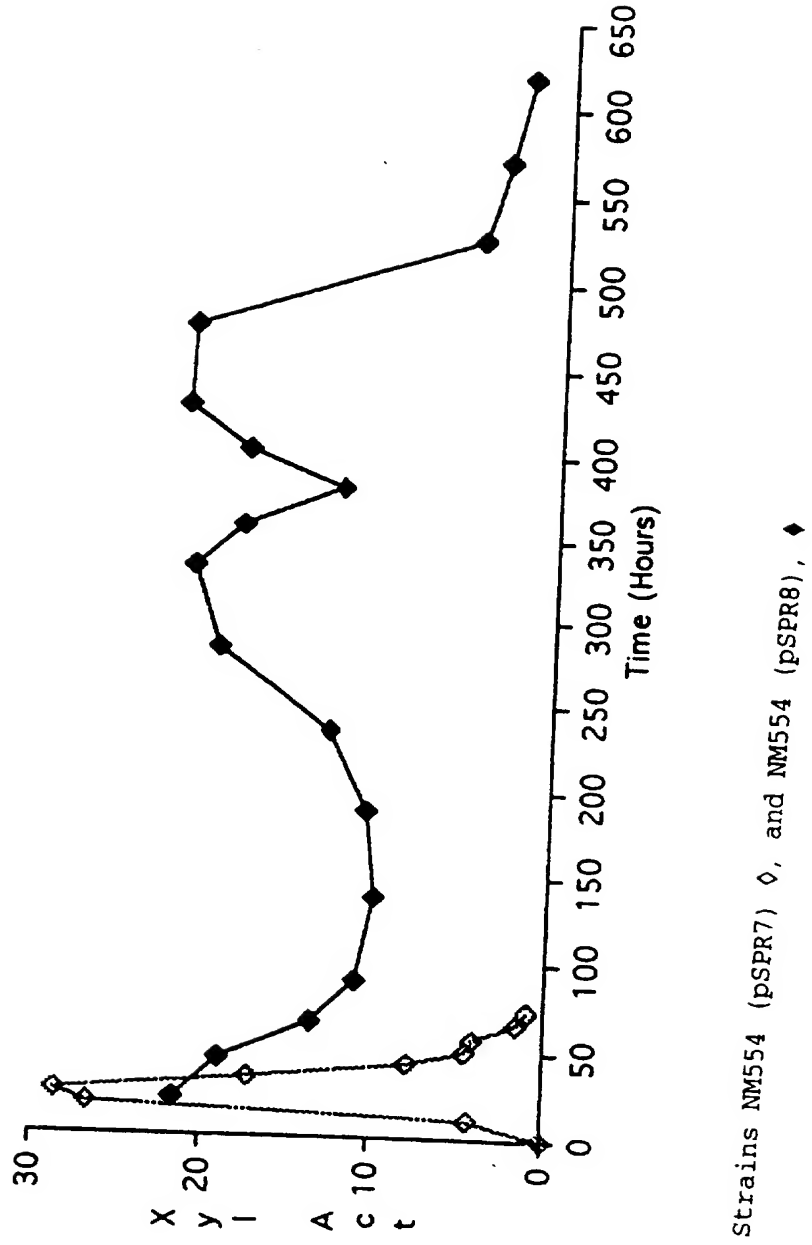
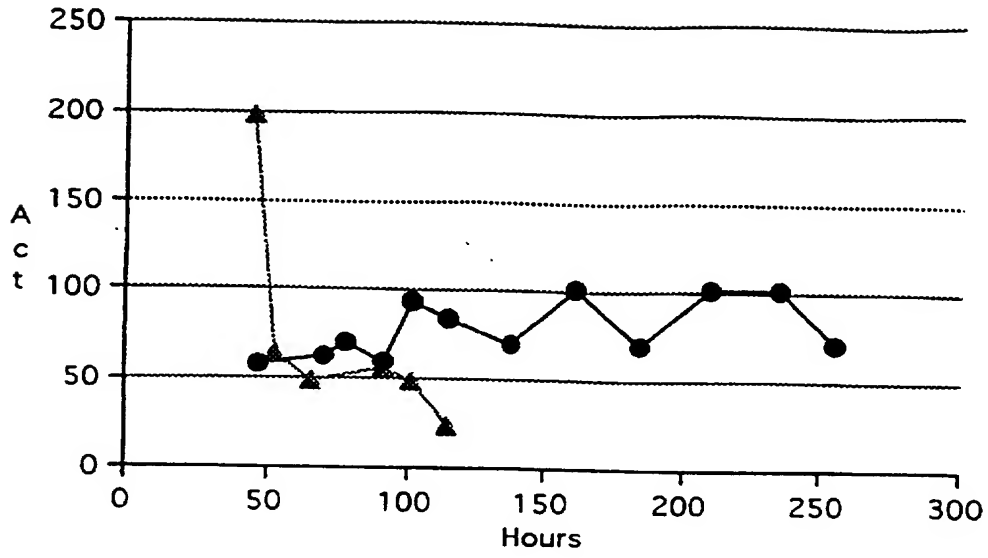

SpeI
6
0
SpeI
5
XYL
ori
1
NotI
pSPR8
6.24 kb
tetA
XYL
2
tetR
4
3
NotI

FIGURE 4 of 5



Strains NM554 (pSPR7) ◊, and NM554 (pSPR8), ◆

FIGURE 5 of 5



INTERNATIONAL SEARCH REPORT

Inte. ›nal Application No
PCT/GB 97/00465

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/68 C12N15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DD 233 852 A (AKADEMIE DER WISSENSCHAFTEN DER DDR) 12 March 1986 see figure 3; examples 1.4,1.5 see page 3, paragraph 3 see page 4, paragraph 2	1-3,5, 8-10, 13-15
Y	---	7
X	US 5 460 954 A (H.W. LEE ET AL) 24 October 1995 see column 3, line 6-39 see column 4, line 59-67 - column 5, line 1-50 see claims 1,2,6,7	1-3,5, 8-10, 13-15

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
23 May 1997	20.06.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Mateo Rosell, A.M.

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INTERNATIONAL SEARCH REPORT

Inte. onal Application No
PCT/GB 97/00465

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BIOCHEMICAL JOURNAL, vol. 272, 1990, LONDON, GB, pages 369-376, XP000576098 L.E. KELLETT ET AL: "Xylanase B and an arabinofuranoside from Pseudomonas fluorescens subsp. cellulosa contain identical cellulose-binding domains and are encoded by adjacent genes" cited in the application see the whole document	7
Y	JOURNAL OF BACTERIOLOGY, vol. 174, no. 8, 1992, WASHINGTON DC, US, pages 2612-2619, XP000197465 P.T: BARTH ET AL.,: "Cloning and partial sequencing of an operon encoding two Pseudomonas putida haloalkanoate dehalogenases of opposite sterospecificity" cited in the application see the whole document	7
A	WO 89 00605 A (CODON) 26 January 1989 cited in the application see page 1, line 7-21 see page 6, line 9 - page 7, line 4 see page 10, line 7-16 see page 12, line 11 - page 13, line 13 see page 18, line 10 - page 19, line 7 see page 22, line 4 - page 23, line 2	1,3,6, 8-10, 12-15
A	US 5 017 488 A (W.T. MCALLISTER ET AL) 21 May 1991 see column 2, line 10-66; claims 1,7	3,9,14, 15
A	JOURNAL OF GENERAL VIROLOGY, vol. 72, no. 12, 1991, READING, GB, pages 2967-2974, XP000197494 U. WEYER AND R.D. POSSEE: "A baculovirus dual expression vector derived from the Autographa californica nuclear polyhedrosis virus polyhedrin and p10 promoters: co-expression of two influenza virus genes in insect cells" see methods and figure 1	1,3,6,8, 9,12-15
A	JOURNAL OF BIOTECHNOLOGY, vol. 8, no. 3, 1988, AMSTERDAM, NL, pages 173-192, XP002031441 P. CAUNT ET AL.: "Stability of recombinant plasmids in yeast" see the whole document	1-15

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. ional Application No
PCT/GB 97/00465

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DD 233852 A		NONE	
US 5460954 A	24-10-95	KR 9509843 B	29-08-95
WO 8900605 A	26-01-89	AU 2263288 A	13-02-89
		EP 0368926 A	23-05-90
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US 5017488 A	21-05-91	US 5037745 A	06-08-91
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